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Supplementary Information for

Antibody cross-reactivity between casein and myelin-associated glycoprotein results in central nervous system demyelination with implications for the immunopathology of multiple sclerosis

Rittika Chunder, Alicia Weier, Hannah Mäurer, Nicolas Luber, Michael Enders, Gabriele Luber, Thorsten Heider, Alfred Spitzer, Sabine Tacke, Janine Becker-Gotot, Christian Kurts, Radhika Iyer, Peggy P. Ho, William H. Robinson, Tobias V. Lanz and Stefanie Kuerten*

*Corresponding author. Prof. Dr. med. Stefanie Kuerten, Institute of Neuroanatomy, Medical Faculty, University of Bonn, 53115 Bonn, Germany; Email: stefanie.kuerten@uni-bonn.de

Supplementary methods

Decomplementation assay

Decomplementation experiments were conducted at the animal facility of the Institute of Anatomy, University of Cologne, Cologne, Germany (approval by the Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV) Nordrhein-Westfalen, 30.11.045). Decomplementation of casein-immunized mice was performed using cobra venom factor (CVF; from the monocled cobra [*Naja kaouthia*]; Merck), an analog of the complement protein C3b, which prevents formation of the terminal C component (2). This triggers activation of the complement cascade in CVF-treated animals, resulting in depletion of C3b from the circulation and therefore the downstream components (C5–C9). Full complement activity is typically restored within two weeks after decomplementation (3). WT B6 mice were injected intraperitoneally with either 50 µg CVF in PBS or PBS alone 10 days after immunization with bovine casein solution. Spinal cord tissues were processed for EM or IHC studies.

Cell cytotoxicity assay

For the cytoxicity assay 100,000 Oli-Neu cells/well were seeded out and differentiated as described earlier. Subsequently, cells were treated with 1% rat serum (Dianova), 100 µg/mL purified casein IgG or both in combination for 24 h. Additionally, an anti-MOG IgG (Novus) and purified anti-mouse IgG (Dianova) were used as a positive and negative control, respectively. A lactate dehydrogenase (LDH) assay was performed following the manufacturer's instructions (ThermoFisher) using 50 µL of cell culture supernatant. The cells were then fixed with 4% PFA and immunstained for proteolipid protein (PLP) for recording of any morphological changes. Images were acquired using a Leica DMi8 inverted microscope (Thunder Imager, Leica). Quantitative analysis of the images was done using NIS Elements (Nikon).

To address what type of cell death is operant in the presence of casein-specific IgG, differentiated Oli-Neu cells were either treated with random IgG, anti-MOG IgG or purified anti-casein IgG (all in combination with 1% rat serum as described above). Cells were then stained with FITC Annexin V Apoptosis Detection kit (BD Biosciences) as per manufacturer's guidelines and data were acquired using a FACSCanto II (BD Biosciences) flow cytometer. Controls for the flow cytometry protocol included single stains of untreated cells, unstained cells and a mixed population of dead and live Oli-Neu cells to determine the gates and for fluoroscence compensation. Analysis was done using the FlowJo software (Tree Star).

Primary cell culture and immunocytochemistry

Primary cultures of oligodendrocyte precursor cells (OPC)s were established using brains from postnatal mice pups between P1 and P3. Briefly, the isolated brains were dissociated using a

gentleMACS™ Octo Dissociator (Milteny Biotec) with appropriate buffers from the Multi Tissue Dissociation Kit (Miltenyi Biotec) according to the manufacturer's instructions. The cell suspension was passed through a cell strainer and washed with 0.5% (v/v) BSA in PBS. After determining the cell number, cells were subsequently resuspended in an appropriate volume of 0.5% (v/v) BSA in PBS and labeled using the CD140a MicroBead kit (Miltenyi Biotec) according to manufacturer's guidelines. Cells were then subjected to magnetic cell sorting using a QuadroMACS™ separator (Miltenyi Biotec). Freshly isolated OPCs were finally cultured in DMEM/F12 medium (ThermoFisher) supplemented with 1% N2 (ThermoFisher), 1% penicillin/streptomycin, 10 ng/mL platelet-derived growth factor (PDGF)-AA (Miltenyi Biotec) and 10 ng/mL fibroblast growth factor (FGF)-2 (Miltenyi Biotec).

For ICC, cells were cultured for 1, 6 or 14 days on 0.001% poly-L-ornithine-coated coverslips at 37 °C and 5% CO_2. Cells were subsequently fixed and stained as described above using antibodies to neural/glial antigen 2 (NG2) and myelin oligodendrocyte glycoprotein (MOG) to study the differentiation of OPCs into mature oligodendrocytes. Quantitative analysis of the images was done using NIS Elements (Nikon).

Furthermore, OPCs that had been cultured for 1 or 14 days were treated with casein- or MOG-specific IgG in combination with 1% rat serum or 1% rat serum alone for 24 h and stained with anti-NG2 antibody to detect morphological changes.

Supplementary figures

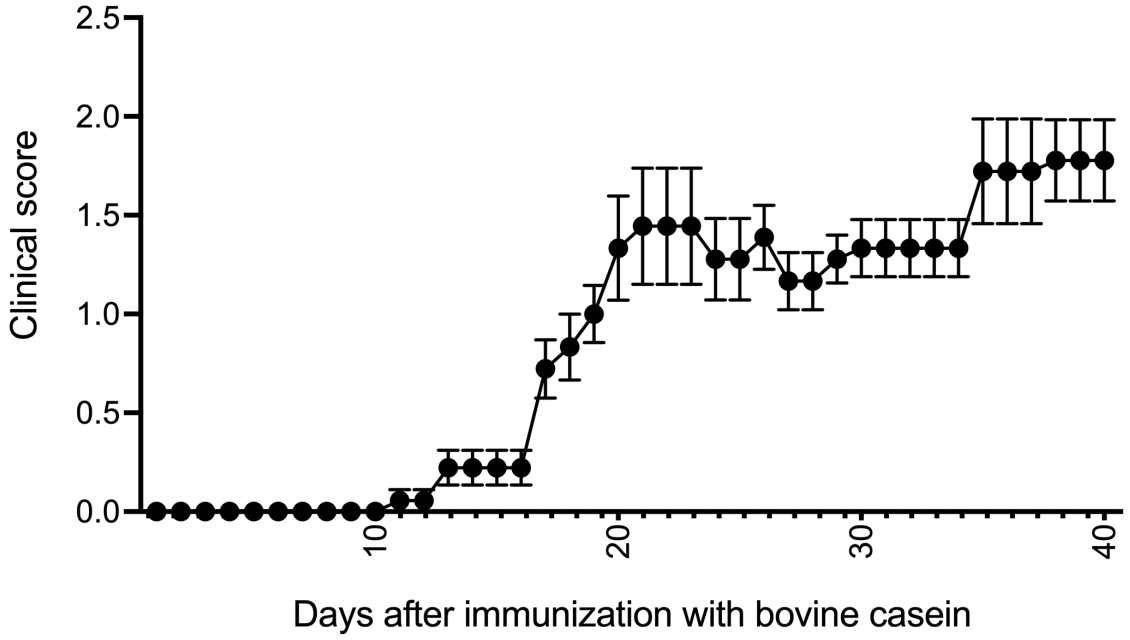

Fig. S1. Clinical course of disease in mice immunized with milk antigens. Graph represents the disease score of mice immunized with bovine casein (*n* = 9) till day 40 after immunization. Every data point represents mean and SEM of the daily score of two consecutive days.

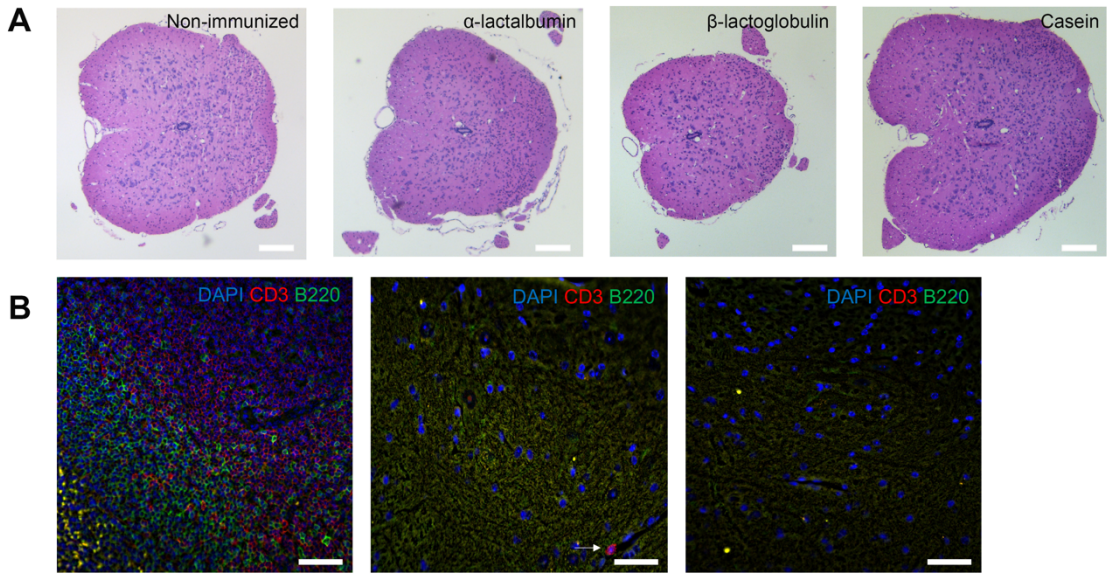

Fig. S2. Screening for immune cell infiltrates in mice immunized with milk antigens. (*A*) Hematoxylin and eosin staining for the detection of inflammatory infiltrates in the lumbar region of the spinal cord. Images are representative of n = 6 – 9 mice per group that were either non-immunized or immunized with α-lactalbumin, β-lactoglobulin or bovine casein. (*B*) CD3 and CD20 staining for the detection of T cell and B cell infiltrates in murine spinal cord sections of casein-immunized mice. The middle and right images are two representative images of two different mice from a cohort of n = 6 mice that were sacrificed at day 40 after immunization with casein. Positive control includes T cell and B cell staining of a murine spleen section (left).

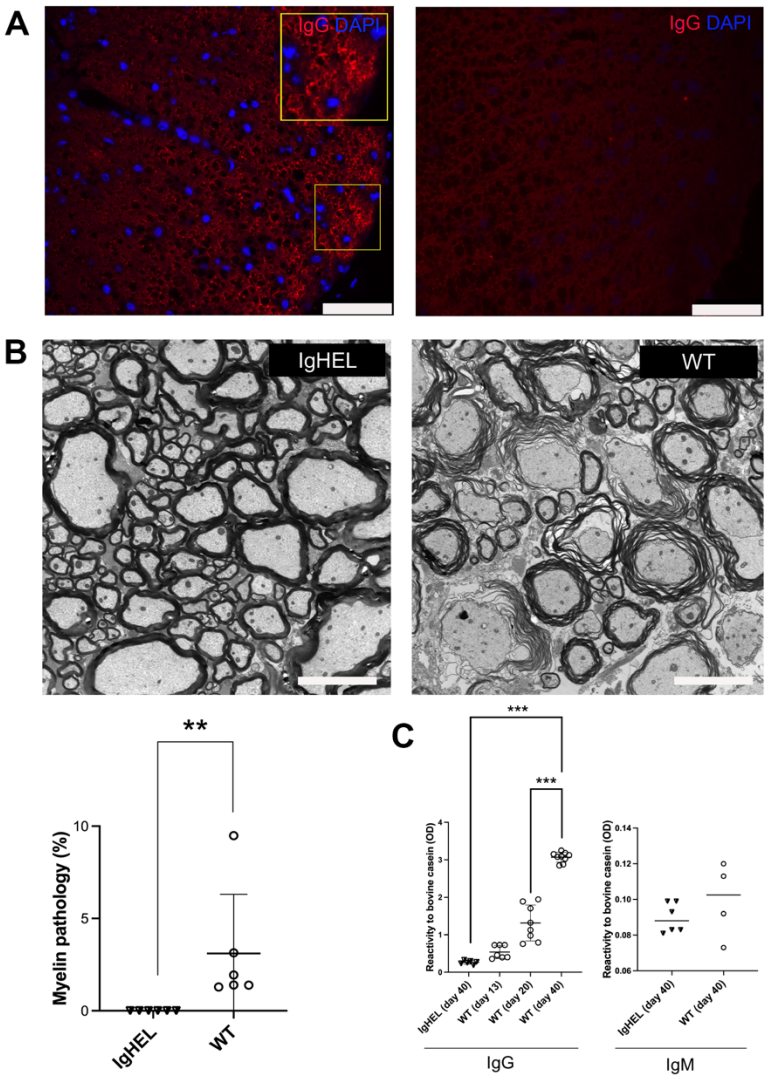

Fig. S3. Spinal cord pathology in casein-immunized WT B6 mice is mediated by casein-specific IgG. (*A*) Ig staining (counterstained with DAPI) on lumbar spinal cord sections from casein-immunized WT mice sacrificed at day 40 (left) versus day 16 (right) after immunization. Scale bars represent 50 µm. (*B*) Spinal cord pathology in casein-immunized IgHEL mice (n = 6) compared with their WT littermates (n = 6), analyzed by electron microscopy. Myelin pathology was quantified by counting the number of demyelinating axons per mouse (from a total of 10 representative images per mouse) as a proportion of the total number of axons. The values represented on the graph are calculated after substracting myelin pathology from healthy mice (negative control) from both groups and negative values were converted to 0. $^{**}p$ = 0.0022 (Mann-Whitney test). (*C*) Casein-specific serum IgM and IgG reactivity measured by ELISA in casein-immunized B6 mice on days 13, 20 and 40 (IgG) and day 40 (IgM) after immunization (n = 4 WT mice for IgM; n = 6 – 9 mice for all other groups). Means ± SD for each group are shown; each data point represents the mean of all OD values from one mouse.

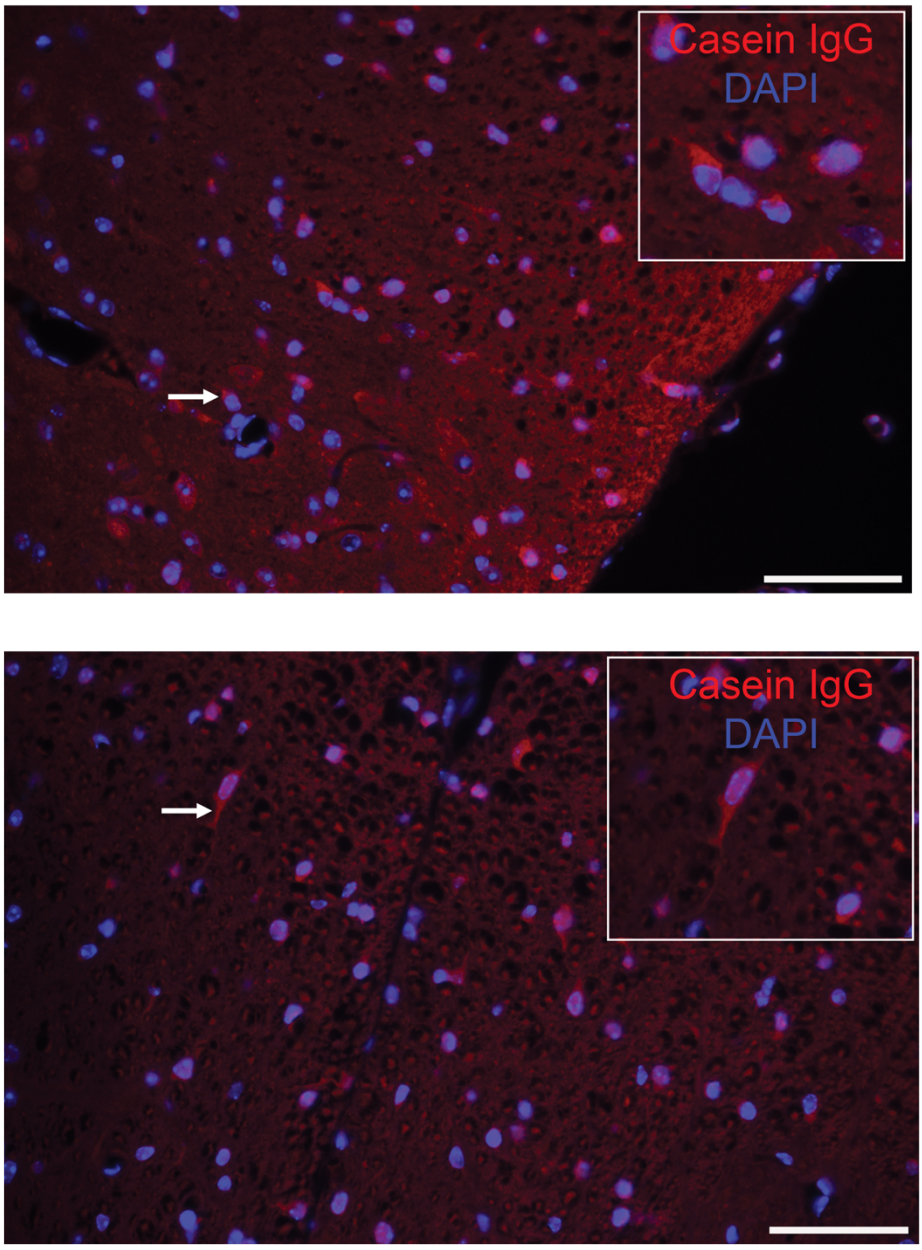

Fig. S4. A range of binding patterns of serum IgG from casein-immunized mice on murine spinal cord. Serum from casein-immunized mice (*n* = 6; day 40) was incubated on spinal cord tissues from non-immunized C57BL/6 mice revealing an array of antigen-antibody binding patterns.

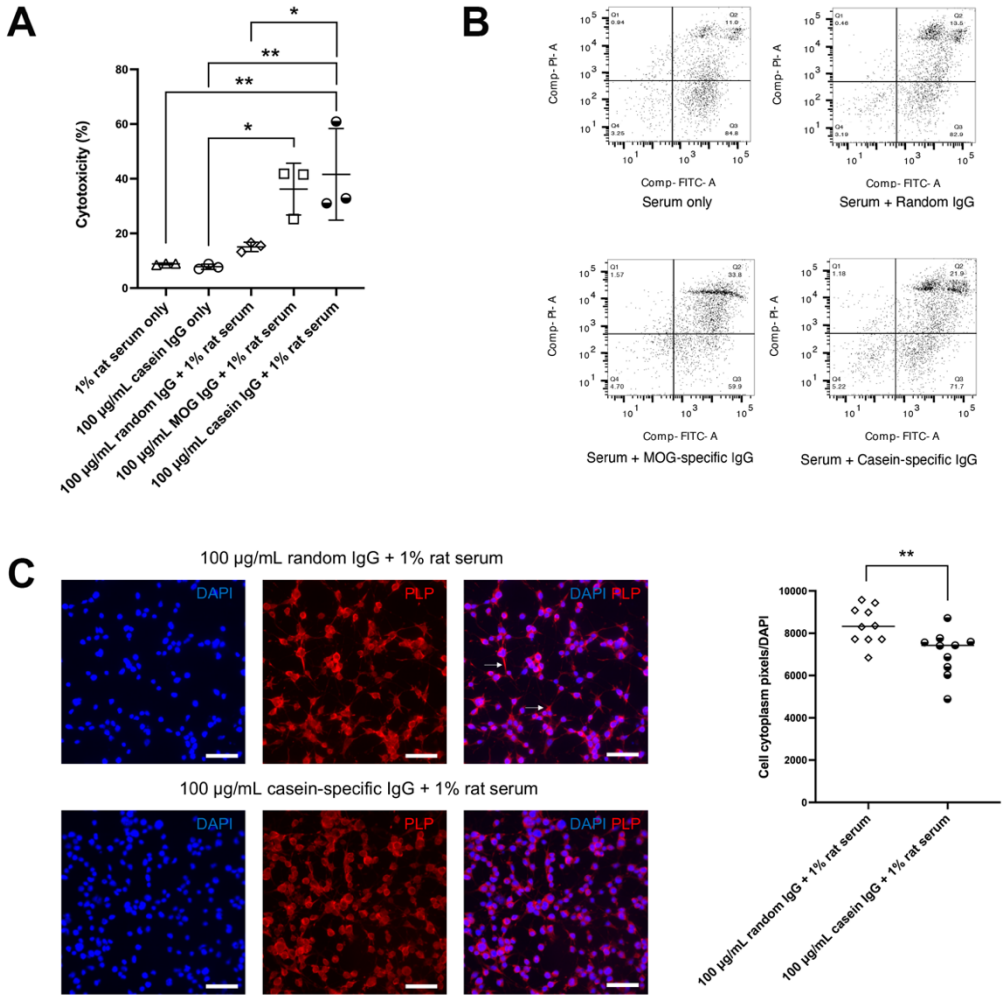

Fig. S5. Complement-dependent cytotoxicity of casein-specific IgG in Oli-Neu cells. (*A*) LDH assay done on differently treated Oli-Neu cells in the presence or absence of antibodies (random IgG, casein-specific IgG and MOG-specific IgG) and 1% serum. ***p* = 0.0052 between 1% rat serum only and casein IgG + 1% rat serum; ***p* = 0.0064 between casein IgG only and casein IgG + 1% rat serum. Means ± SD for each group are shown; each data point represents the mean of three technical replicates. (*B*) Differentiatied Oli-Neu cells treated either with 100 µg/mL casein-specific IgG, MOG-specific IgG or random IgG and 1% rat serum were stained for annexin V (FITC) and PI and the apototic cell population was detected by flow cytometry (Q2). The gates were determined by single staining of Oli-Neu cells using only PI or annexin V (FITC). (*C*) Differentiated Oli-Neu cells treated with either 100 µg/mL of random IgG or casein IgG and 1% rat serum stained for PLP. Arrows in the upper panel indicate the presence of oligodendroglial processes when Oli-Neu cells were treated with random IgG and rat serum. These processes, however, became less prominent in the presence of casein IgG and rat serum (lower panel). Scale bars represent 50 µm. The ratio between the pixel count of the cytoplasm and nucleus is shown. ***p* = 0.0082 (unpaired *t* test).

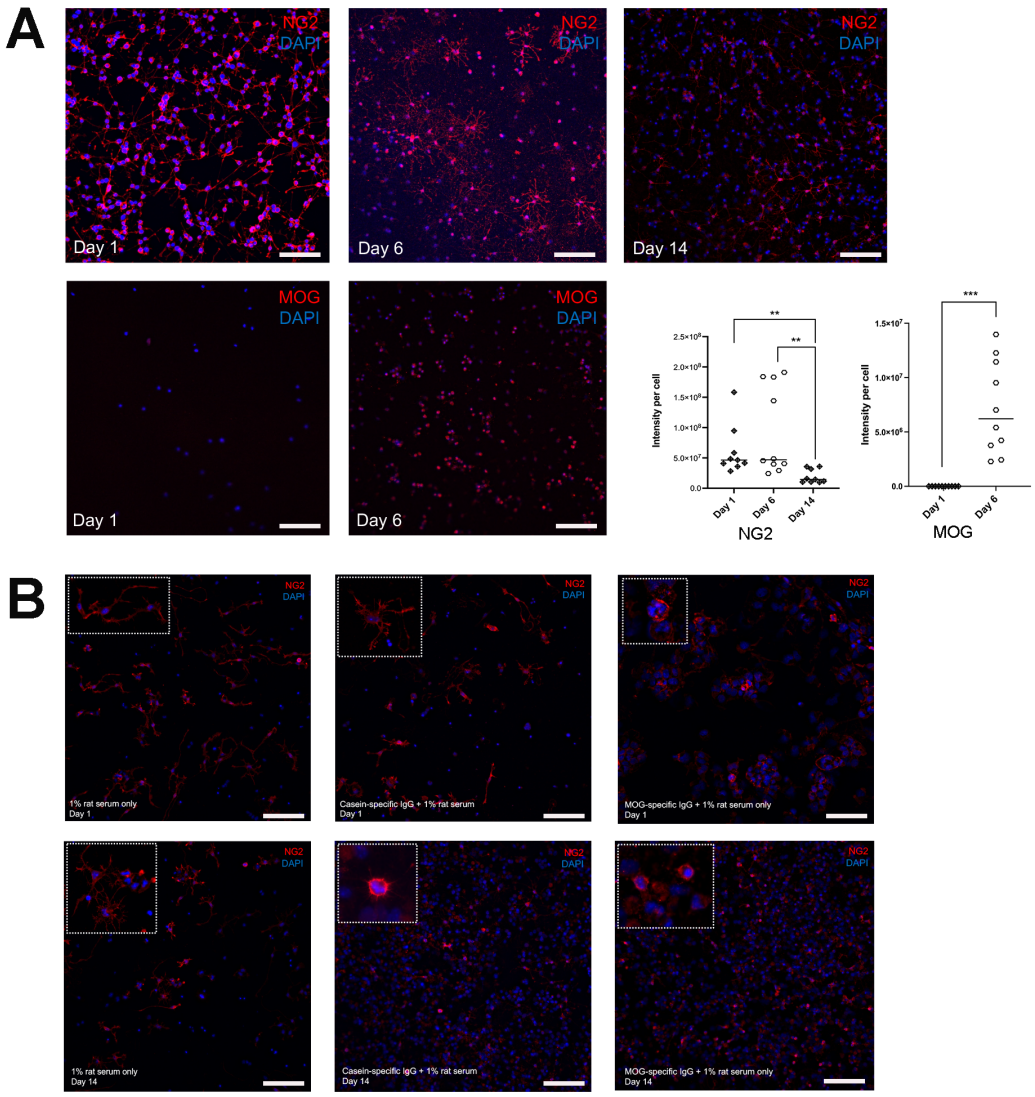

Fig. S6. Complement-dependent morphological changes induced by casein-specific IgG in undifferentiated and differentiated primary OPCs. (*A*) Analysis of primary OPC differentiation using NG2 and MOG as maturation markers. Cells were stained on culture days 1 (NG2, MOG), 6 (NG2, MOG) and 14 (NG2) (***$p < 0.0001$ for MOG day 1 vs. 6; **$p = 0.0065$ for NG2 day 1 vs. 14 and **$p = 0.0021$ for NG2 day 6 vs. 14). (*B*) OPCs on culture days 1 and 14 were treated either with 1% rat serum only or in combination with 100 µg/mL casein- or MOG-specific IgG. Morphological changes are displayed. The scale bars represent 50 µm.

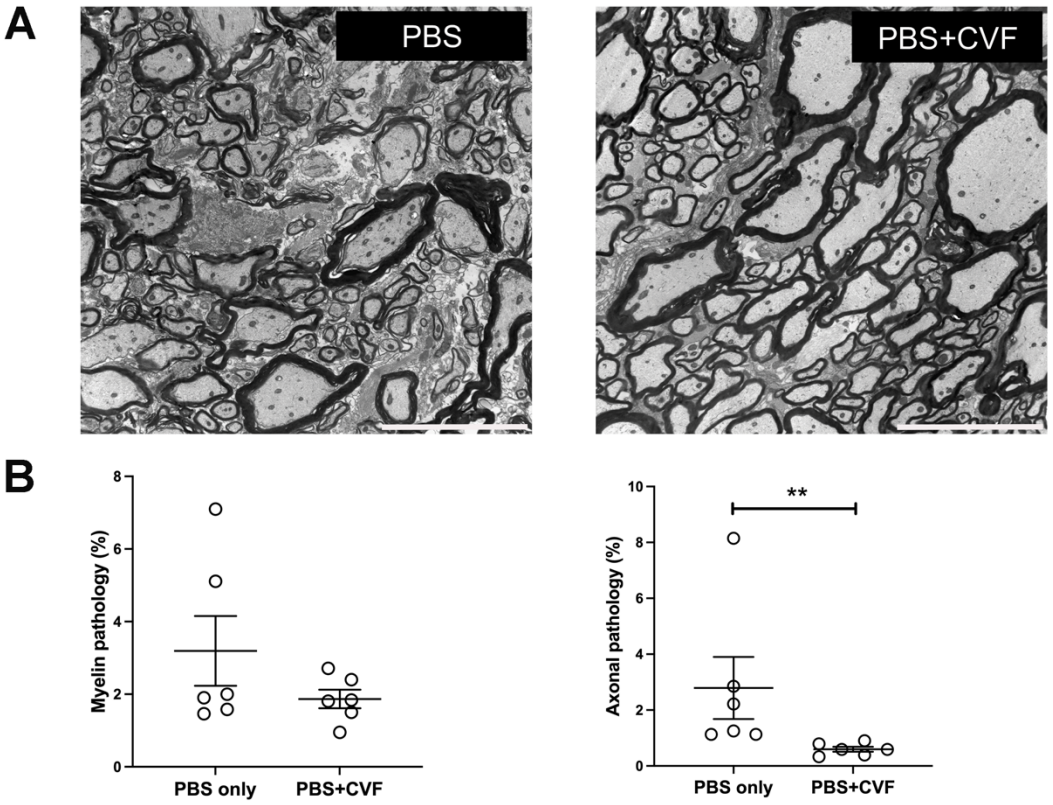

Fig. S7. Transient depletion of the complement system results in decreased spinal cord pathology in casein-immunized WT B6 mice. (*A*) Representative images of spinal cord pathology in casein-immunized WT B6 mice treated with either CVF (i.e., complement-depleted) or PBS (control) 10 days following casein immunization. Scale bars represent 5 μm. (*B*) Spinal cord pathology was evaluated by electron microscopy and the percentage of myelin pathology ($p = 0.17$) and axonal pathology (**$p = 0.002$) compared between the two groups (unpaired *t* test). Demyelinating pathology was quantified by calculating the number of demyelinated axons per mouse (from a total of 10 representative images per mouse). Axonal pathology was similarly quantified by counting the number of damaged axons. Means with SEM for each group are shown.

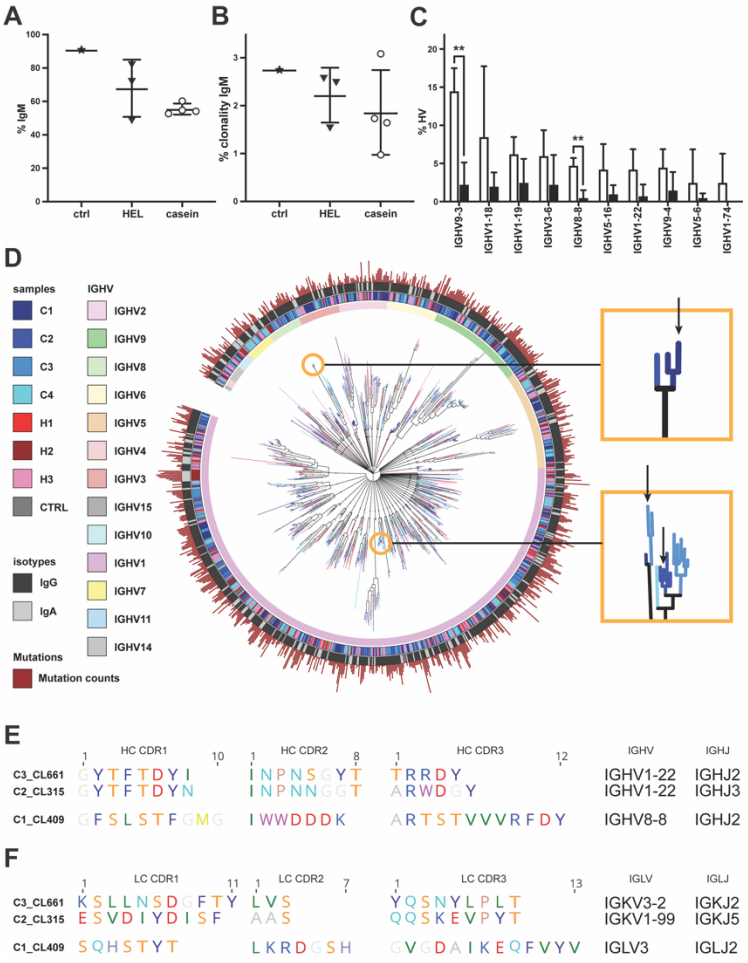

Fig. S8. Repertoire analysis between casein- and MAG-specific antibodies reveals cross-reactivity. (*A* and *B*) Repertoire analyses, separated for the three groups of casein- (n = 4) and HEL-immunized mice (n = 3), as well as the control mouse (ctrl, n = 1). (*A*) Number of IgM sequences, represented as percentage of all sequenced antibody sequences. (*B*) Clonality of IgM sequences, bar and whiskers represent means ± SD for each group; each data point represents mean of all respective sequences from one mouse. (*C*) Selected IGHV genes that were over-represented in casein-immunized mice vs. HEL and control mice; $^{**}p < 0.005$, multiple *t* tests with correction for multiple hypothesis testing according to Benjamini, Krieger and Yekutieli. (*D*) Phylogenetic tree of all IgG and IgA sequences. Innermost circle indicates IGHV group, second circle as well as branch color indicates mouse from which antibody sequence was derived, third circle indicates immunoglobulin class (IgG or IgA), bar graph in the outermost circle indicate mutation counts. Circles and inserts indicate clusters containing the casein/MAG binding antibody sequences, corresponding to Fig. 3, *G* to *I*. (*E* and *F*) CDR amino acid sequences of the three casein/MAG binding mAbs. (*E*) Alignment of HC CDR regions and corresponding IGHV/IGHJ names. (*F*) Alignment of LC CDR regions and corresponding IGLV/IGLJ names.

Supplementary tables

Table S1. Expression of the different casein genes in the CNS of healthy mice[a]

Gene	Ct		ΔΔCt	Fold change
	Brain	**Breast**		
CSN1S1	22.91	16.29	6.62	0.016
CSN2	22.74	16.31	6.43	0.019
CSN3	22.20	10.61	11.59	0.00045

[a]ΔΔCt values and fold change were calculated using β-actin as housekeeping gene and breast tissue as a positive control.

Table S2. Amino acid sequence homologies between murine MAG and bovine caseins

Target sequence	Query sequence	Identity[a]	Residues overlap	Amino acid start position
Mouse MAG	Bovine αS1-casein	50%	12	MAG_{543} Cas_{189}
		44.4%	9	MAG_{602} Cas_{58}
		60%	5	MAG_{57} Cas_{177}
		50%	8	MAG_{194} Cas_{177}
		100%	3	MAG_{23} Cas_{177}
		66.7%	6	MAG_{109} Cas_{44}
		50%	8	MAG_{495} Cas_{185}
		60%	5	MAG_{293} Cas_{84}
	Bovine β-casein	70%	10	MAG_{496} Cas_{70}
		57.1%	7	MAG_{268} Cas_{153}
		43.8%	16	MAG_{235} Cas_{20}
		80%	5	MAG_{143} Cas_{97}
		44.4%	9	MAG_{64} Cas_{194}
	Bovine κ-casein	42.9%	7	MAG_{182} Cas_{112}
		75%	4	MAG_{146} Cas_{177}
		50%	8	MAG_{109} Cas_{57}
		57.1%	7	MAG_{433} Cas_{173}
		66.7%	9	MAG_{498} Cas_{13}
		41.2%	17	MAG_{544} Cas_{166}

[a]Identity overlaps less than 40% are not listed in the table.

CASA1, αS1-casein; CASB, β-casein; CASK, κ-casein.

Table S3. Characteristics of patients with multiple sclerosis

Patient No.	Subtype of MS	Sex	Age	Time since diagnosis (Y)	EDSS Score	Daily Consumption				
						Whole Milk (mL)	Skim Milk (mL)	Butter Milk (mL)	Cheese (g)	Dairy Yoghurt (g)
MS 1	SPMS	F	63	32	5.4	0	0	0	20	150
MS 2	RRMS	F	54	12	3.0	0	0	0	0	200
MS 3	RRMS	M	29	11	2.5	250	0	0	10	50
MS 4	RRMS	M	52	22	3.0	35.7	0	0	42.85	7.14
MS 5	RRMS	M	49	11	1.5	500	0	500	10	0
MS 6	RRMS	F	24	9	1.0	250	0	0	21.4	21.4
MS 7	RRMS	M	56	24	2.5	0	28.57	0	14.28	21.4
MS 8	SPMS	F	60	17	4.0	0	0	0	28.57	0
MS 9	RRMS	F	20	2	2.0	–				
MS 10	RRMS	F	40	4	3.0	0	71	0	28.57	85.7
MS 11	RRMS	F	48	18	3.0	142.85	0	0	0	57.14
MS 12	RRMS	F	51	5	2.0	0	71.4	0	7.14	71.4
MS 13	RRMS	F	33	1	1.5	0	28.57	71.4	7.14	142.85
MS 14	RRMS	F	52	5	2.5	0	200	0	10	200
MS 15	RRMS	M	45	5	3.0	0	0	0	0	7.14
MS 16	RRMS	F	45	11	3.0	14.28	0	0	14.28	0
MS 17	RRMS	F	24	1	2.5	4.28	0	0	17.14	0
MS 18	RRMS	F	55	14	5.5	1000	0	0	200	0
MS 19	SPMS	M	59	29	5.0	300	0	0	100	20
MS 20	SPMS	M	56	27	3.5	250	0	0	0	0
MS 21	RRMS	F	55	3	1.5	0	0	0	14.28	28.57
MS 22	RRMS	M	33	1	1.5	0	300	0	50	200

Patient No.	Subtype of MS	Sex	Age	Time since diagnosis (Y)	EDSS Score	Daily Consumption				
						Whole Milk (mL)	Skim Milk (mL)	Butter Milk (mL)	Cheese (g)	Dairy Yoghurt (g)
MS 23	RRMS	M	35	8	2.0	0	71.4	0	71.4	0
MS 24	RRMS	F	48	20	2.5	0	0	0	14.28	14.28
MS 25	RRMS	F	41	0.75	2.0	0	14.28	0	42.9	21.42
MS 26	RRMS	M	38	10	1.0	28.57	0	0	14.28	14.28
MS 27	RRMS	F	57	14	1.5	0	0	0	7.14	150
MS 28	RRMS	M	33	1	1.0	0	0	0	0	0
MS 29	RRMS	F	48	3	3.0	0	20	0	14.28	75
MS 30	RRMS	M	37	1	1.0	0	200	100	50	150
MS 31	RRMS	F	39	2	3.5	0	500	0	2.85	50
MS 32	RRMS	F	63	25	1.5	50	0	100	0	150
MS 33	SPMS	M	54	25	4.5	35.7	0	0	71.42	0
MS 34	RRMS	F	54	19	2.0	0	142.85	0	0	71.4
MS 35	RRMS	F	51	16	2.0	71.4	0	0	14.28	0
MS 36	RRMS	F	37	20	2.5	60	0	250	7.14	42.9
MS 37	RRMS	F	29	3	1.5	500	0	0	4.28	14.28
MS 38	RRMS	F	58	33	2	0	0	0	0	200
MS 39	RRMS	F	70	17	3	0	0	0	0	0
MS 40	RRMS	F	45	1	2	0	0	0	14.28	42.85
MS 41	Unknown	F	34	0.5	3.0	Not available				
MS 42	Unknown	F	29	12	2.0	Not available				
MS 43	Unknown	F	49	4	2.0	Not available				
MS 44	Unknown	F	49	5	1.0	Not available				
MS 45	Unknown	F	54	7	5.0	Not available				

EDSS, expanded disability status scale; *F*, female; *M*, male; *MS*, multiple sclerosis; *RRMS*, relapsing-remitting multiple sclerosis; *SPMS*, secondary progressive multiple sclerosis; *PPMS*, primary progressive multiple sclerosis.

Table S4. Characteristics of patients with other neurological diseases (controls)

Patient No.	Disease	Sex	Age	Daily Consumption				
				Whole Milk (mL)	Skim Milk (mL)	Butter Milk (mL)	Cheese (g)	Dairy Yoghurt (g)
C 1	Parkinson's disease	F	84	0	0	400	14.28	0
C 2	Psychosis, epilepsy, hydrocephalus, optic nerve atrophy	M	48	0	150	0	28.57	150
C 3	Carotid artery stenosis, carpal tunnel syndrome	M	82	0	0	0	20	26.7
C 4	Optic neuritis, migraine	F	56	0	60	0	7.14	0
C 5	Depression, vestibular schwannoma, anxiety disorder, spinal stenosis	F	88	0	500	0	50	100
C 6	Parkinson's disease, polyneuritis, polyneuropathy	M	78	30	0	0	30	100
C 7	Neuralgia, facial-sparing scapular myopathy, allodynia	M	57	500	0	0	20	0
C 8	Polyneuropathy	M	64	0	0	0	21.4	21.4
C 9	Multifocal motor neuropathy	F	46	0	0	0	1.4	14.28

Patient No.	Disease	Sex	Age	Daily Consumption				
				Whole Milk (mL)	Skim Milk (mL)	Butter Milk (mL)	Cheese (g)	Dairy Yoghurt (g)
C 10	Depression	M	55	142.85	0	0	7.14	250
C 11	Depression, OCD	M	41	100	0	0	50	50
C 12	Dementia, vitamin B12 deficiency	F	85	14.28	0	0	14.28	28.57
C 13	Bipolar disorder	F	49	0	0	0	7.14	14.28
C 14	Epilepsy	M	25	42.85	0	0	21.4	0
C 15	Epilepsy	M	53	0	0	0	0	100
C 16	Multifocal motor neuropathy	M	55	20	0	0	21.4	100
C 17	Nerve contusions	M	48	0	500	142.9	0	0
C 18	Tremor, Parkinson's disease	M	68	0	0	0	0	10.71
C 19	Depression	M	40	0	0	0	4.3	100
C 20	Epilepsy	M	81	500	0	0	10	100
C 21	Myasthenia gravis	F	24	285.7	0	0	0	0
C 22	Psychosomatic problems	F	59	0	71.42	0	21.4	150
C 23	Psychosis, vascular headache	F	53	0	0	0	0	100
C 24	Depression, migraine	F	56	0	250	250	20	300
C 25	Cyst of the pineal gland	F	26	0	250	0	4.28	75

Patient No.	Disease	Sex	Age	Daily Consumption				
				Whole Milk (mL)	Skim Milk (mL)	Butter Milk (mL)	Cheese (g)	Dairy Yoghurt (g)
C 26	Psychosomatic problems, ischemic stroke	F	63	0	0	0	2.8	28.6
C 27	Depression, psychosis	F	59	0	400	0	14.28	42.85
C 28	Carpal tunnel syndrome	F	64	0	0	0	0	200
C 29	Bipolar disorder	F	46	50	0	0	100	0
C 30	Unknown	F	44	100	0	0	60	150
C 31	OCD, depression	F	54	0	0	0	0	0
C 32	Polyneuropathy	F	58	0	0	0	20	0
C 33	Parkinson's disease, depression	F	79	200	0	0	28.57	35.7
C 34	Psychosomatic problem, lumbago, migraine	F	41	0	0	0	0	75
C 35	Bipolar manic depression	F	71	14.28	0	0	125	100

C, control; *F*, female; *M*, male; *OCD*, obsessive compulsive disorder.

Table S5. Immunization cohorts of mice

Strain of mouse	Immunization (milk antigens)	Number of mice/ cohort	Sacrificed on	Method used for
C57BL/6 WT	Casein	*n* = 9	Day 13	EM, IHC, ELISA (time kinetics)
		n = 9	Day 20	
		n = 9	Day 40	
		n = 6		IHC, adsorption assay
		n = 6		
		n = 10	Day 60	ICC, cytotoxic assay
		n = 18	Day 16	Complement depletion assay, EM, IHC
	β-lactoglobulin	*n* = 5	Day 40	EM, IHC
	α-lactalbumin	*n* = 4		
	Casein	*n* = 4	Day 31	Antibody repertoire sequencing
	HEL	*n* = 3		
IgHEL	Casein	*n* = 6	Day 40	ELISA, EM, IHC

Table S6. Gene list and primer pairs

Gene	Forward Primer 5′→ 3′	Reverse Primer 5′→ 3′	Reference
Myelin oligodendrocyte glycoprotein (*MOG*)	GACCTCAGCTTGGCCTGACCC	TGCTGGGCTCTCCTTCCGC	4
Myelin-associated glycoprotein (*MAG*)	CTCTATGGCACCCAGAGCCT	TGTCCTTGGTGGGTCGTTTT	5
Myelin basic protein (*MBP*)	ATGGCATCACAGAAGAGACC	CATGGGAGATCCAGAGCGGC	6
Proteolipid protein (*PLP*)	AGCGGGTGTGTCATTGTTTGGGAA	ACCATACATTCTGGCATCAGCGCA	7
αS1-casein (*CSN1S1*)	TGACCAGTCAGTGATGATGTC CAGTCAGTGATGATGTCATGCTT	ACTGTGGAGTTCCTGAGAGA TGTAGAATTTTGCTCTCCGTGT	Self-designed
β-casein (*CSN2*)	TGAGATACCCAAGCTGCACA TCCGTTTCTGTCTAAGAGGATTTC	TGCAGCTGAAGTCTGAGTGTAG CATTTCCAGTTTCAGTCAGTTCA	Self-designed
κ-casein (*CSN3*)	CAAACCCTACTGCCAAGCAAG	TTGTAGGCATGGCAAGAAAGG	8

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